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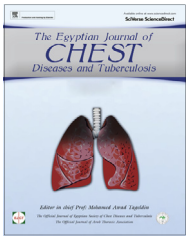

ORIGINAL ARTICLE

Evaluation of urinary glycosaminoglycans and matrix metalloproteinase-7 in lung cancer patients

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Abstract Lung cancer accounts for one-third of all cancer-related deaths worldwide. Glycosaminoglycans and matrix metalloproteinase-7 (MMP-7) have been found to be involved in cancer. The aim of our study was to evaluate the clinical significance of urinary glycosaminoglycans and MMP-7 in lung cancer patients in order to explore their potential diagnostic utility and predicting ability with respect to the different clinicopathological parameters.

Urine samples used in the assay of glycosaminoglycans and MMP-7 were collected from 70 lung cancer patients (45 squamous cell lung cancer and 25 adenocarcinoma). Control subjects were 15 age and sex matched non smokers.

The malignant group mainly adenocarcinoma type showed significant increase in urinary glycosaminoglycans and MMP-7 compared to control group. Regarding the different clinicopathological factors, urinary glycosaminoglycans and MMP-7 showed significant increase with the histological grades and clinical stages ($p < 0.05$, $p < 0.05$ respectively). The best cut-off values for glycosaminoglycans and MMP-7 determined by Receiver operating characteristic (ROC) curve were 67.5 μg/mg creatinine and 10.05 ng/mg creatinine respectively. The sensitivity and specificity were 91.4% and 86.7% for glycosaminoglycans and 71.4% and 86.7% for MMP-7. The combined assay of the two parameters raised the overall positivity rate to 88.9% and 94.7% regarding grade 1 and stage I respectively.

In conclusion, our findings can indicate that urinary glycosaminoglycans and MMP-7 can be potential non invasive helpful markers in lung cancer patients.

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Introduction

Lung cancer has become the number one killer among cancers worldwide [1].

In spite of aggressive therapy available today, the prognosis of lung cancer patients is generally very poor due to lack of biomarkers for the reliable detection of malignant lung tumors

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[2]. Therefore, the development of novel diagnostic markers to identify lung cancer is important to facilitate earlier diagnosis of primary or recurring cancers thus leading to more effective treatment and improved prognosis [3].

The extracellular matrix (ECM) not only provides tissue support, but its functional macromolecules are also involved in the regulation of cell properties and function. This is mainly due to interactions between ECM components and cell-surface receptors, growth factors and cytokines. Therefore, ECM constituents are closely involved in the cellular and molecular mechanisms of cancer cells, and affect their adhesion and migration as well as their invasiveness and metastatic potential. Proteoglycans, which make up a major part of the ECM, are heavily glycosylated glycoproteins. The specific type of polysaccharides attached to proteoglycans are called glycosaminoglycans (GAGs). GAGs, are long, linear and highly charged, heterogeneous polysaccharides that are composed of a variable number of repeating disaccharide units and most of them, as their name implies, have a sweet taste. The GAGs are involved in signaling cascades regulating angiogenesis, invasion and metastasis of malignant cells [4].

In the lung, GAGs support the structure of the interstitium, the subepithelial tissue and the bronchial walls, and are secreted in the airway secretions. Besides maintaining lung tissue structure, GAGs also play an important role in lung function as they regulate hydration and water homeostasis, modulate the inflammatory response and influence lung tissue repair and remodeling [5].

Metalloproteinases are secreted proteins belonging to a family of zinc metalloendopeptidases that have the capacity to cleave ECM. They can modify both cell–cell and cell–ECM interactions by the proteolysis of cell surface growth factors and adhesion receptors. Because their substrates are diverse, metalloproteinases are involved in a variety of homeostatic functions, such as bone remodeling, wound healing, and several aspects of immunity. However, metalloproteinases are also involved in a number of pathological processes, such as tumor progression, fibrosis, chronic inflammation and tissue destruction [6]. Matrix metalloproteinase-7 (MMP-7) is the smallest one of the matrix metalloproteinases. It has been found that MMP-7 is highly expressed in glandular epithelial cells and carcinoma of cells of epithelial origin and that it promotes cell migration [7].

In cancer, the study of GAGs [8] and MMP-7 [9] has been primarily twofold: first, in aiding diagnosis and second, in developing alternative treatments. Therefore, this study was conducted to evaluate the clinical significance of urinary GAGs and MMP-7 in lung cancer patients, also their potential diagnostic utility and predicting ability with respect to the different clinicopathological parameters.

Materials and methods

Patients and tissue samples

This study has complied with the principles laid down in the Declaration of Helsinki, 1964. The studied groups included 70 lung cancer patients (45 squamous cell lung cancer and 25 adenocarcinoma patients). Fifty eight were males and 12 were females. Their ages ranged from 41 to 75 years old (61 ± 7.2). Fifteen age matched (62 ± 8) and sex matched non smoker adults were also collected and acted as the control. The control subjects presented with hemoptysis ($n = 8$), with chronic persistent cough ($n = 3$) with no obvious evident explanations, with foreign body aspiration ($n = 2$) and 2 subjects with postoperative lobe or segmental atelectasis (therapeutic bronchoscope). Tissue samples were obtained from all subjects who underwent fiberoptic bronchoscopy in Ain Shams University Hospitals in Cairo, Egypt. Patients did not receive any form of therapy before bronchoscopy. Specimens were fixed in 10% formalin solution for histological examination. Lung cancer was staged using a widely used classification system and graded by the Nottingham grading system [10].

The urinary levels of GAGs and MMP-7 in the malignant group were compared with the clinicopathological characteristics, including smoking status, histological grade and TNM stage.

Procedure of fiberoptic bronchoscopy and bronchial biopsy

Patients and controls were prepared for elective fiberoptic bronchoscopy. Written consent was obtained after a reassuring explanation of the steps of bronchoscope. Prior to the procedure, the general condition and vital signs as well as arterial blood gas and pulmonary function test were assessed. Exclusion of any active ischemic heart disease, severe respiratory insufficiency even on supplemental oxygen and coagulation disorders was done. They were fasting for at least 6 hours before the procedure and three hours after to avoid any possible complications. It is usual to give the patient supplemental oxygen via nasal cannula and to monitor the saturation by pulse oximetry.

Atropine sulphate 0.6 mg was given intramuscularly 30 min before the procedure to reduce bronchial secretions and to block a possible vasovagal reflex. About 20–40 min after premedication (atropine sulphate), a 4% lidocaine (lignocaine) sprayed into both nostrils and the patient's mouth. Having anaesthetized the upper respiratory tract, the shaft of the bronchoscope is well lubricated with 2% lidocaine gel and is advanced into a nostril under direct vision, through the widest visible opening between the turbinates. If the bronchoscope does not advance easily it should be withdrawn and the other nostril tried, if failed, mouth approach should be tried. As the instrument is advanced, the tip is flexed downwards and the epiglottis and larynx come into view. The position and movement of the vocal cords with respiration is noted and the patient is asked to say "eeee", so that full apposition of the cords can be observed and vocal cord paralysis confirmed or excluded. Additional 2.5 ml aliquots of 2% lidocaine (50 mg) may be instilled down the suction channel of the bronchoscope to facilitate passage of the bronchoscope through the vocal cords to the trachea and trachea. Tracheobronchial mucosa is normally smooth and pink, the main carina and subcarinae (bronchial or segmental spurs) are usually sharp. Thorough bronchoscopic evaluation we can localize endobronchial masses.

A centrally placed tumor may be clearly visible as an endobronchial mass of tissue that may partially or totally occlude the lumen in which it is situated. Loss of the usual mucosal luster and presence of roughened surface with ulceration and necrosis of the overlying mucosa may alert the expert bronchoscopist to an early infiltrative or neoplastic process.

Confirmation is provided by biopsy of the suspected areas followed by histopathological examination.

Bleeding may occur during biopsy, this is an encouragement to the bronchoscopist to spray the suspected lesion with a bolus of 5 ml 1:20000 epinephrine (adrenaline) solution before taking another repeated biopsy.

Urine samples collection and processing

Each participant was asked to void early morning urine sample in a sterile container. Each urine sample was centrifuged at 3000 rpm for 25 min and the supernatant was divided into two aliquots stored at −80 °C until used; one without preservative used for measurement of MMP-7 using MMP-7 Quantikine ELISA kit (R&D Systems Inc., Minneapolis, USA) and creatinine using creatinine Biolabo kit (BIOLABO SA, Maizy, France) according to manufacturers' instructions and the second aliquot was preserved by the immediate addition of methanol (0.1 ml methanol/10 ml urine) used for measurement of glycosaminoglycans.

Quantitative determination of urinary glycosaminoglycans

Urinary glycosaminoglycans were precipitated according to the method of Ferrante [11], and adapted by Pennock [12]. Twenty five milliliters of each preserved sample was centrifuged at 3000 rpm for 25 min and 5 ml of supernatant was precipitated with 10% cetylpyridinium chloride purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. The precipitate was collected by centrifugation, washed twice with 95% ethanol containing 10% potassium acetate and dissolved in 1 ml of 0.05 M NaOH. Glycosaminoglycans were determined in urine according to the previously described method of Whitley et al. [13], using chondroitin sulfate sodium salt (from shark cartilage, no. immunoassay C4384) purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany as a standard. Briefly, a 10X stock solution (0.35 mmol/L) of 1,9-dimethylmethylene blue (DMB) dye (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was made by dissolving 122 mg of dye in 10 ml of 95% ethanol which was then diluted to1 liter with sodium formate buffer (pH 3.5, 0.2 mmol/L). 40 µl of each urine sample (or standard) was mixed with 1.0 ml of freshly prepared working DMB dye (1X). The absorbance was measured after 30 min incubation at 535 nm against buffer. The measured values were corrected for reagent blank and sample blank absorbances. Glycosaminoglycan concentrations (µg/ml) were determined from the standard curve then normalized to urinary creatinine and expressed in µg GAGs/mg creatinine.

Statistical analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19). Data are expressed as mean ± standard deviation (SD). The studied parameters were related to the clinicopathological parameters using ANOVA test. Correlation between variables was performed by Pearson test. For the above comparisons, $p < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curve determined the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true positive fraction (sensitivity percent) and false positive fraction (100-specificity) of markers at several cut off points.

Results

The studied groups included 70 lung cancer patients (45 squamous cell lung cancer and 25 adenocarcinoma patients) and 15 lung tissue samples from non smoker control adults and their clinicopathological factors are shown in Fig. 1 and Table 1.

Clinical sample analysis

Urinary concentrations of GAGs and MMP-7 were measured in both the malignant and control groups (Tables 2 and 3). The mean urinary GAGs and MMP-7 increased significantly in the malignant group (125.2 ± 46.7 and 14.0 ± 5.1 respectively) versus the control group (40.8 ± 15.6 and 5.8 ± 2.1 respectively, $p < 0.01$) Table 2.

The mean levels increased significantly in adenocarcinoma patients compared to squamous cell carcinoma patients (168.1 ± 36.6 and 102 ± 33.5 respectively for urinary GAGs, 17.4 ± 5.7 and12.1 ± 4.7 respectively for MMP-7, $p < 0.01$), Table 3.

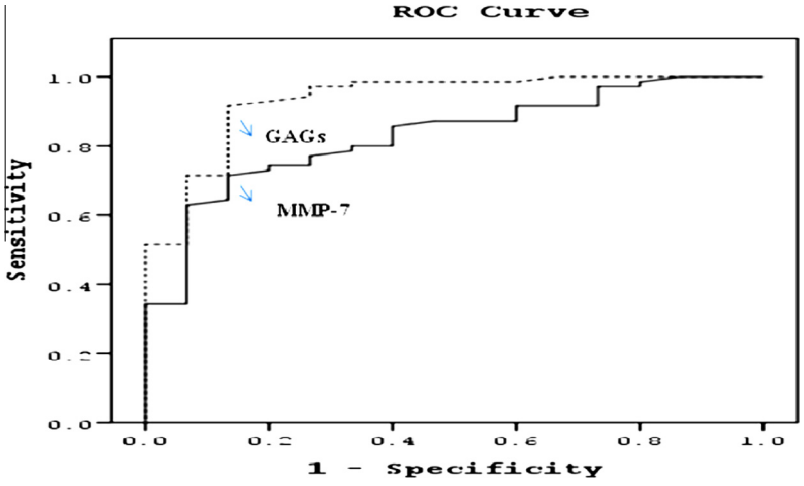

Figure 1 ROC curve of urinary GAGs and MMP-7 in lung cancer patients.

Table 1 Clinicopathological factors in different study groups.

Clinicopathological factors	Control No. (%)	Malignant No. (%)	
		Squamous cell lung carcinoma	Adenocarcinoma
Sex			
Male	12 (80%)	38 (54.3%)	22 (31.4%)
Female	3 (20%)	7 (10%)	3 (4.3%)
Non smoker	–	19 (27.1%)	4 (5.7%)
Smoker		26 (37.2%)	21 (30.0%)
Affected lung			
Right lung	–	29 (41.4%)	18 (25.7%)
Left lung		16 (22.9%)	7 (10%)
Affected lobe			
Upper lobe	–	29 (41.4%)	15 (21.4%)
Middle lobe		11 (15.7%)	3 (4.3%)
Lower lobe		5 (7.2%)	7 (10%)
Grade; No. (%)			
Grade 1	–	7 (10%)	2 (2.9%)
Grade 2		30 (42.9%)	8 (11.4%)
Grade 3		8 (11.4%)	15 (21.4%)
Stages; No. (%)			
Stage I	–	16 (22.9%)	3 (4.3%)
Stage II		13 (18.5%)	11 (15.7%)
Stage III		16 (22.9%)	11 (15.7%)
Tumor size			
< 3 cm	–	28 (40%)	10 (14.3%)
> 3 cm		17 (24.3%)	15 (21.4%)

Table 2 Urinary levels of GAGs and MMP-7 in the malignant group compared to the control group.

Parameter	Control group (mean ± SD)	Malignant group (mean ± SD)
GAGs (μg/mg creatinine)	40.8 ± 15.6	125.2 ± 46.7**
MMP-7 (ng/mg creatinine)	5.8 ± 2.1	14.0 ± 5.1**

** $p < 0.01$ is highly significant;
** $p < 0.01$ versus control group.

Cut-off points for urinary GAGs and MMP-7

Optimal cut-off points were 67.5 μg/mg creatinine and 10.05 ng/mg creatinine for urinary GAGs and MMP-7 respectively. The best cut-off values were estimated to maximize the sum of sensitivity and specificity, Table 4.

The relation between investigated marker and clinicopathological factors

The levels of urinary GAGs and MMP-7 in relation to clinicopathological characteristics are shown in Table 5. There was no significant relation between both parameters and smoking status ($p > 0.05$). However, There was significant relation between investigated parameters and the different histological grades and clinical stages ($p < 0.05$). The positivity rate of combined assay of both parameters (number of cases having urinary level of GAGs and/or MMP-7 higher than the cut-off value of the corresponding parameter) was 88.9%, 89.5% and 91.3% in grade 1, 2 and 3 respectively, while it was 94.7%, 91.7% and 92.6% in stage I, II and III respectively.

Table 3 Urinary levels of GAGs and MMP-7 in squamous cell carcinoma patients compared to adenocarcinoma patients.

Parameter	Squamous cell carcinoma group (mean ± SD)	Adenocarcinoma group (mean ± SD)
GAGs (μg/mg creatinine)	102 ± 33.5	168.1 ± 36.6**
MMP-7 (ng/mg creatinine)	12.1 ± 4.7	17.4 ± 5.7**

** $p < 0.01$ is highly significant;
** $p < 0.01$ versus squamous cell carcinoma.

Correlation between investigated parameters

Concerning the level of the investigated parameters to each other, there was a significant positive correlation ($r = 0.544$), Table 6.

Discussion

The investigation of the role that GAGs and MMP-7 plays in cancer has been the focus of much recently done studies.

Previous reports showed significant increase of the urinary GAGs excretion in patients with bladder cancer compared to normal subjects [14,15] and significant increase of serum GAGs levels in lymphoma patients compared to control subjects [16]. As regards lung, studies demonstrated an increase of GAGs excretion in urine of asthmatic subjects [17,18]. In lung cancer, reports demonstrated an increase in the total amount of GAGs in serum of patients of lung cancer compared to that of healthy subjects [19], also reported an increase of GAGs in lung cancer tissues compared to normal lung tissues and that this increase was accompanied by changes in the composition of GAGs [20]. Serum and tissue expression levels of MMP-7 were found to be elevated in many cancers like prostatic [21] and gastric [22]. In lung cancer MMP-7 was found to be expressed by lung tumor cells of all histological types at various frequencies [23,24] but to our knowledge no study has been done to evaluate the clinical role of urinary GAGs and MMP-7 in lung cancer diagnosis and progression. Therefore, urinary levels of GAGs and MMP-7 in 70 lung cancer patients and 15 healthy age-matched subjects were estimated in this study. Our results showed that the mean urinary GAGs (μg/mg creatinine) and MMP-7 (ng/mg creatinine) was

Table 4 Sensitivity and specificity of urinary GAGs and MMP-7 by using receiver operating characteristic (ROC) curve.

Parameter	Cut-off	Sensitivity (%)	Specificity (%)	Area under ROC curve
GAGs (μg/mg creatinine)	67.5	91.4	86.7	0.933
MMP-7 (ng/mg creatinine)	10.05	71.4	86.7	0.828

Table 5 Relation between GAGs and MMP-7 urinary levels and clinicopathological factors.

Factors	Urinary GAGs		Urinary MMP-7		Urinary GAGs and MMP-7
	Mean ± SD	Positivity rate	Mean ± SD	Positivity rate	Overall positivity rate
Smoking					
Non smokers ($n = 23$)	99.47 ± 32.84	18 (78.3%)	11.6 ± 5.5	15(65.2%)	21 (91.3%)
Smokers ($n = 47$)	141.64 ± 47.21	42 (89.4%)	15.1 ± 3.6	35(74.5%)	45 (95.7%)
Grade					
Grade 1 ($n = 9$)	77.6 ± 25.52	7 (77.8%)	10.8 ± 3.9	6 (66.7%)	8 (88.9%)
Grade 2($n = 38$)	107.93 ± 32.32	33 (86.8%)	12.8 ± 4.9	27 (71%)	34 (89.5%)
Grade 3 ($n = 23$)	170.77 ± 34.56 $p < 0.05^*$	20 (87%)	17.2 ± 5.1 $p < 0.05^*$	17 (73.9%)	21 (91.3%)
Stages					
Stage I ($n = 19$)	110.91 ± 30.94	15 (78.9%)	12.7 ± 4.2	13 (68.4%)	18 (94.7%)
Stage II($n = 24$)	111.23 ± 37.24	21 (87.5%)	11.6 ± 5.1	16 (66.7%)	22 (91.7%)
Stage III($n = 27$)	149.93 ± 55.79 $p < 0.05^*$	24 (88.9%)	17.1 ± 4.3 $p < 0.05^*$	21 (77.8%)	25 (92.6%)

* $p < 0.05$ is significant.

Table 6 Correlation between the investigated parameters.

		MMP-7
GAGs	Pearson correlation	0.544*

* Correlation is significant at $p < 0.05$ level.

increased significantly in the malignant group (125.2 ± 46.7 and 14.0 ± 5.1 respectively) versus the control group (40.80 ± 15.6 and 5.8 ± 2.1 respectively, $p < 0.01$). We found also that the mean level increased significantly in adenocarcinoma patients compared to squamous cell carcinoma patients (168.1 ± 36.6 versus 102 ± 33.5 for urinary GAGs and 17.4 ± 5.7 versus 12.1 ± 4.7 for urinary MMP-7, $p < 0.01$). This result was in agreement with previous reports [23,24] which demonstrated that the expression of lung tumor cells for MMP-7 was significantly higher in adenocarcinoma type.

Regarding the different clinicopathological factors, the mean urinary levels of GAGs and MMP-7 showed significant increase with the different histological grades and clinical stages ($p < 0.05$, $p < 0.05$ respectively) which was in agreement with many reports which found significant correlation between the urinary levels of GAGs and the different stages and grades of bladder cancer [14,15]. The highest mean levels of investigated parameters were detected in grade 3 and stage III compared to grades (1 and 2) and stages (I and II). We also found a significant positive correlation between urinary GAGs and MMP-7 ($r = 0.544$). The combined measurement of urinary GAGs and MMP-7 increased the positivity rate for lung cancer in respect to the different stages and grades.

Conclusion

Our findings suggested that urinary GAGs and MMP-7 increased significantly in lung cancer patients mainly in adenocarcinoma type and with the different histological grades and clinical stages of the disease. They also showed high sensitivity and specificity as well as high positivity rates as regards the different clinicopathological parameters especially with combined measurement of the two parameters. This can indicate that urinary GAGs and MMP-7 can be potential non invasive helpful markers in lung cancer which provide a new biochemical approach for detecting and monitoring the pathogenesis of lung cancer and ultimately, improve patient's prognosis and quality of life. Further studies on a large cohort of patients will be necessary to confirm these findings, also the types and structural changes of excreted GAGs in the urine of lung cancer patients should be investigated for the development of alternative lung cancer therapy.

Conflict of interest

The authors have no conflicting interests, including any financial, personal or other relationships with other people or organizations, and are not supported or funded by any company.

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